In Vitro Cell. Dev. Biol.—Animal 33:479–482, June 1997

1071-2690/97 $05.00+0.00

DIFFERENTIAL REQUIREMENTS OF TWO INSECT CELL LINES FOR GROWTH IN SERUM-FREE MEDIUM

J. L. VAUGHN AND F. FAN

Insect Biocontrol Laboratory, Plant Sciences Institute, ARS, USDA, Beltsville, Maryland 20705

(Received 26 April 1996; accepted 16 July 1996)

SUMMARY

The development of a serum-free medium that supports the growth of cells from a *Spodoptera frugiperda* and a *Lymantria dispar* cell line is reported. A yeast hydrolysate provided the B-vitamin complex, and a combination of a meat hydrolysate and tryptose provided most of the free amino acids required for cell growth. Supplemental cystine and methionine were required to achieve maximum cell growth. The serum or serum replacements used in earlier formulations were replaced with commercial lipid preparations and increased levels of iron salts. Although the cell growth cycle had a somewhat extended lag phase and the population doubling time of the *S. frugiperda* cells was longer than on serum-containing medium, the saturation densities were much higher.

Spodoptera cells grown in this medium replicated the *Autographa californica* nuclear polyhedrosis virus well, producing 8.71×10^6 $TCID_{50}$ extracellular virus and 4.4×10^8 polyhedra/ml culture. The specific activity of the polyhedra was somewhat less than that of polyhedra produced in insects.

Key words: Spodoptera; Lymantria; gypsy moth; lipids; hydrolysates; virus; *Autographa californica*; nuclear polyhedrosis.

INTRODUCTION

The success of the baculovirus expression vector system for producing foreign gene products in insect cells has greatly increased interest in methods for their culture. As a result, there has been active commercial development of specialized, protein-free media for insect cells, and a number of tissue culture supply firms sell one or more formulations of these media, primarily for the culture of the Sf9 line from *Spodoptera frugiperda*. These media support cell growth to high saturation densities and high levels of foreign gene expression in pilot scale bioreactors.

The successful development of methods for the large-scale insect cell culture has renewed the interest in the commercial *in vitro* production of baculoviruses for insect pest management. However, production costs, which include media costs, are a more important factor in the commercial acceptance of cell culture for the production of viruses than for the production of gene products. Thus, there is a need for inexpensive media that will provide results similar to those with the protein-free media. Gardiner and Stockdale (1975) developed a simplified medium which consisted of inorganic salts, lactalbumin hydrolysate, tryptose broth, and water soluble vitamins. This medium supported growth of the *S. frugiperda* cell line, Sf-21, but only limited virus replication and still required supplementation with 10% fetal bovine serum. Gardiner and Stockdale also observed that although their medium would support cell growth and limited virus replication, there was poor incorporation of virions into the occlusion bodies, indicating that nutrition adequate for cell growth would not necessarily support complete virus replication and assembly.

Vail et al. (1976) reported the assembly of infectious occlusion bodies in the cabbage looper cell line, Tn-368. In their serum-free medium, infectivity of the inclusion bodies produced was normal, but the numbers were reduced by almost one-half after the cells had been passed 44 times on the medium.

Goodwin and Adams (1980) developed a basic serum-free medium containing a mixture of amino acids and peptones that supported some growth of gypsy moth cell lines but no replication of the baculovirus. The addition of the synthetic peptide glycyl-L-histidyl-lysine improved the cell growth but not the virus replication. Removing the lactalbumin hydrolysate and increasing the level of TC Yeastolate in the peptone half of the combination resulted in regular incorporation of virions into the occlusion bodies. Further modification of the formula by adding methyl oleate, cholesterol, α-tocopherol, α-glycerophosphate, and glycerol supported serial passage of the gypsy moth cell line with the shortest population doubling time of any of their serum-free media. Virus replication was not determined in cells passaged in this medium.

We recently reported a medium formulation for a serum-free medium that supported good growth of *S. frugiperda* and *Lymantria dispar* cells (Vaughn and Fan, 1991). This formulation, Medium VIII, contained the inorganic salt mixture found in the IPL-41 medium of Weiss et al. (1981), glucose, a yeast hydrolysate, peptones, cystine, methionine, inosine, and choline. Final cell densities of the CL-15 clone of a *S. frugiperda* line reached 9×10^6 cells/ml when the basal medium was supplemented with a commercial lipid preparation. The *L. dispar* cell line, LDFB, grew to final densities of 2.25×10^6 cells/ml when the medium was supplemented with lipids plus the serum replacement CPSR-3 (Sigma Chemical Co., St. Louis, MO).

This paper reports further attempts to optimize the medium for each of these cell lines and to obtain growth of LDFB cells in medium without the serum replacement.

Materials and Methods

Cell lines. The cell lines used in this study were: Cl-15, a clone obtained from the *S. frugiperda* line IPLB-Sf-21AE (Vaughn et al., 1977) and IPLB-Ld-FB from *L. dispar* (Lynn et al., 1988). Identity of the cell lines was confirmed by isozyme analysis with the Authentikit™ (Innovative Chemistry, Marshfield, MA) as described by Lynn and Hung (1991). Stock cultures were maintained on Medium VIII supplemented with lipids (Cl-15) or lipids and CPSR-3 (LDFB). Transfers to experimental formulations were made without special adaptation. Data on growth were not collected until the final cell densities had stabilized, but never until there had been at least three passages on the test formulation.

Culture conditions. The studies were conducted in 100-ml suspension cultures in 250 disposable Erlenmeyer flasks (Corning Glass, Corning, NY). The cells were protected from shear forces by the addition of 1 gm of Pluronic F-68/L (BASF Corp., Parsippany, NJ). Test cultures were inoculated at 0.3×10^6 cells/ml and incubated in air at 28° C on an orbital shaker operated at 180 rpm. We determined growth by removing three 0.1-ml samples from each flask at each sample time and counting in a hemocytometer. Viable cells were identified by trypan blue exclusion.

Virus studies. Frozen stocks from the third *in vitro* passage of *Autographa californica* multiple embedded nuclear polyhedrosis virus (AcMNPV) were used to test the ability of cells growing in the IBL10 medium to support viral replication. Cl-15 cells in 15 ml of medium in 125-ml disposable Erlenmeyer flasks (Corning, No. 25605-125) were inoculated during the log phase of growth at a multiplicity of infection of 0.01. The inoculated culture was incubated on a rotating shaker at 180 rpm at 28° C. Seven days postinfection the culture was harvested and centrifuged at 500 ×g for 10 min. The supernatant was removed and frozen until the extracellular virus (ECV) could be assayed. The pellet was resuspended in distilled water and sonified with a Heat Systems-Ultrasonics, Inc. (Plain View, NY) cup sonicator until the cells were disrupted and a uniform suspension of polyhedra was obtained. Polyhedra were counted with an AO Bright-Line Hemocytometer and phase-contrast optics.

Polyhedra assay. Specific activity of the polyhedra produced was determined by a feeding assay with 5-day-old *Trichoplusia ni* larvae. Polyhedra were diluted in distilled water, and 1 ml of each dilution was added to the surface of a 180-ml diet container. Ten larvae were added to a cup and three cups were used for each dilution. The larvae were incubated at 29° C, 50% relative humidity, and a photoperiod of 12:12 (L:D) until all larvae in the controls had pupated. Mortality regressions were estimated with LeOra Software (1987).

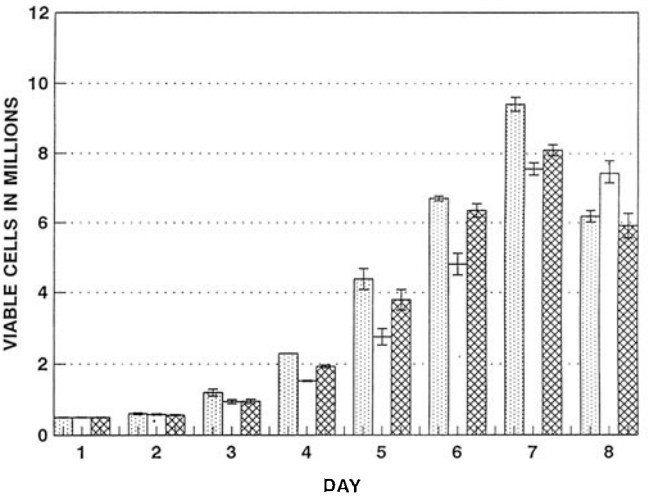
ECV assay. Assays were done in 96-well tissue culture plates (Falcon, No. 3072). Each well was seeded with 0.1 ml of IPL-41 medium supplemented with 10% CPSR-3 (Sigma, No. C 9155) containing 4×10^4 cells/ml. The virus suspension was serially diluted in the same medium and 12 wells inoculated with 0.1 ml of each dilution. Controls received 0.1 ml of additional medium. The plates were incubated at room temperature in a moist chamber for 7 days and then examined for the presence of polyhedra in each well. The $TCID_{50}$ and the $TCID_{50}$s per ml were estimated with a BASIC computer program with Karbers method for calculating 50% endpoints.

Results

Cell Growth. Based upon the product profiles supplied by the manufacturer, the mixture of Primatone, an enzymatic hydrolysate of meat, and Amisoy, an acid hydrolysate of soy (Sheffield Products, Norwich, NY) used in our medium, supplied most of the free amino acids in quantities equal to or exceeding those in Medium IPL-41. The exceptions were cystine and methionine which had to be added separately. However, finding that combinations of hydrolysates provided better growth than any individual hydrolysate led us to test additional combinations. Liver extract, Peptone, and Tryptose (Oxoid, Ogdensburg, NY) in combination with Primatone were selected for this study. These hydrolysates are commonly used in culture media for microorganisms, are inexpensive, and available in bulk quantities.

The Peptone–Primatone combination supported the least growth of either cell line (Figs. 1 and 2). The most striking difference in growth response of the two cell lines was to the liver–Primatone combination. The gypsy moth cells grew only slightly better in this combination than in Peptone–Primatone. The Cl-15 cells grew to a saturation density of 9×10^6 cells/ml in seven days, better than in any other combination. The population doubling time was 43.63 h. The Tryptose–Primatone combination supported the best growth of the LDFB cells. They reached a saturation density of almost 5×10^6 cells/ml in six days with a population doubling time of 31.78 h. The Cl-15 cells reached a saturation density of slightly more than 8×10^6 cells/ml with a population doubling time of 43.24 h.

Product profiles of the serum replacements provided by Sigma Chemical indicated that one of the significant differences between CPSR-3, which supported LDFB cell growth, and CPSR-1, which did not, was the level of lipids. Also Goodwin and Adams (1980) had



FIGS. 1–2. Growth of cells from *Spodoptera frugiperda* and *Lymantria dispar* cell lines in media containing combinations of hydrolysates. *Bar* and *line* represents the mean and SD of three samples per test. *Stippled bar*, Primatone–Liver extract; *open bar*, Primatone–Peptone, and *cross-hatched bar*, Primatone–Tryptose.

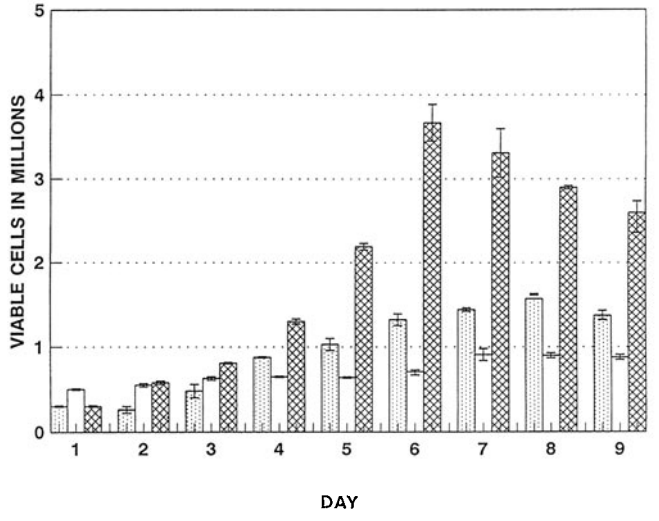


FIG. 2

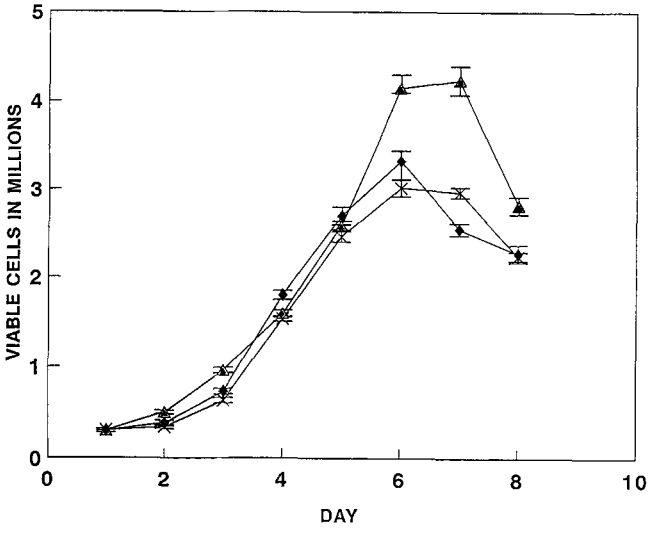


FIG. 3. The growth of gypsy moth cells, IPL-LDFB, in Medium IBL10 supplemented with CPSR-3 plus 0.1% Lipid Medium Supplement (–◆–); 0.2% Lipid Medium Supplement (–×–); or 0.4% Lipid Medium Supplement (–▲–). *Lines* represent SD of mean cell number of three samples per test.

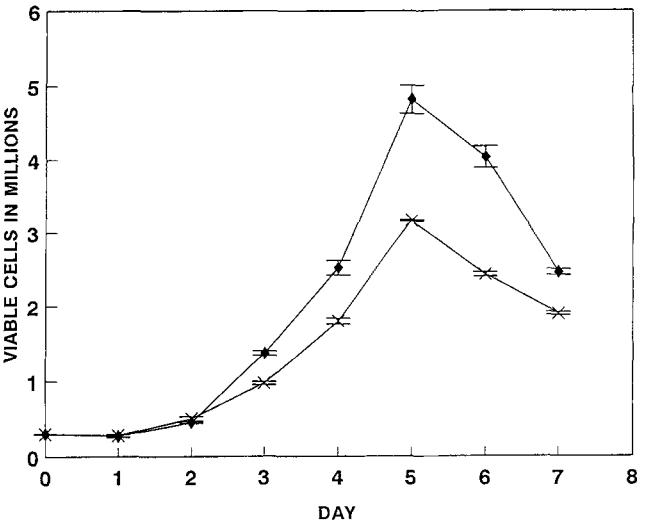


FIG. 4. The effect of increased iron levels on the growth of gypsy moth, LDFB, cells. Iron at 0.55 mg/L (–×–) and at 2.20 mg/L (–◆–). *Lines* represent SD of mean cell number of three samples per test.

demonstrated the importance of lipids for good insect cell growth. Thus, attempts to replace CPSR-3 in the medium for the LDFB cells began with studies on effects of additional lipid on cell growth. We found that increasing the lipid level to 0.2% vol/vol with the Lipid Medium Supplement (Sigma Chemicals, St. Louis, MO) supported a saturation density nearly equal to that in medium containing 5% CPSR-3 and 0.1% lipid (Fig. 3). Further increasing the level of lipids to 0.4% vol/vol resulted in a saturation density of just over 4×10^6 cells/ml in 5 days.

The last modification was the level of iron in the basal medium. Although iron had never been demonstrated to be essential for insect cell growth, it is present in all serum-containing media. We increased the level of iron from 0.55 mg/L to 2.2 mg/L. As shown in Fig. 4, the saturation density was increased to about 5×10^6 cells/ml and the population doubling time reduced to 26.67 h.

The formulation of the resulting medium, Medium IBL10, is given in Table 1. In addition to the two cell lines used in the development of this medium, a third cell line, IPLB-PxE-5AIII, from the diamondback moth, (*Plutella xylostella*) has been adapted to it. A saturation density approaching 20×10^6 cells/ml (data not shown) was achieved in early passages of this cell line.

Virus replication. Preliminary studies show that the spodopteran cells grown in this medium will support complete replication of an infectious nuclear polyhedrosis virus. The amount of ECV produced, 8.71×10^6 $TCID_{50}$ units/ml, was equal to that produced normally in this lab with the uncloned cell line and IPL-41 medium supplemented either with fetal bovine serum or serum replacements (Vaughn et al., 1991). Polyhedra production in IBL10 medium, 8.8×10^7/ml, was equal to that reported for the IPL-41 medium plus serum replacements. However, the specific activity (4.6×10^3 polyhedra/LC_{50} unit) of the polyhedra produced in the IBL10 medium was about one-third that of polyhedra produced in *T. ni* larvae (Martin Shapiro, personal communication).

TABLE 1

FORMULATION OF MEDIUM IBL10[a]

Components	mg/L	Components	mg/L
$NaH_2PO_4 \cdot H_2O$	1,160	Glucose	10,000
$NaHCO_3$	350	Yeastolate	10,000
KCl	1,200	Tryptose	5,000
$CaCl_2$	500	Primatone	5,000
$MgSO_4 \cdot 7\ H_2O$	1,880	Cystine	50
$(NH_4)Mo_7O \cdot 4\ H_2O$	0.04	Methionine	250
$CoCl_2 \cdot 6\ H_2O$	0.05	Inosine	400
$CuCl_2 \cdot H_2O$	0.20	Choline chloride	20
$MnCl_2 \cdot 4\ H_2O$	0.02	Pluronic F-68	1,000
$ZnCl_2$	0.04		
$FeSO_4 \cdot 7\ H_2O$	2.20		

[a]Lipid Medium Supplement (Sigma Chemical Co., St. Louis, MO); 0.4% vol/vol Mannitol added to adjust the osmotic pressure to 350 mOsm/kg; pH adjusted to 6.3.

DISCUSSION

This study shows that a low-cost, serum-free medium based upon hydrolysates will support growth of insect cells to high saturation densities. Supplementation of two amino acids, cystine and methionine, was critical to the success of this medium. Both have been shown to be essential for the growth of insect cells (Mitsuhashi, 1989). The importance of the lipids to insect cell growth was further documented in this study, especially in the response of the LDFB cells to lipid concentration in the serum-free medium. The lipid supplement provided cholesterol, D-α-tocopherol acetate, and fatty acids and methyl esters from cod liver oil. These compounds have been shown to be important for the replication of the NPV (Goodwin and Adams, 1980). The lipid preparation also contains polyoxyethylenesorbitan monooleate (Tween 80) as an emulsifier, but the role of this material on virus replication is not known. Further studies are

needed to identify optimum levels of these compounds to improve the specific activity of the polyhedra produced. The effect of the concentration of iron was unexpected. To our knowledge this is the first demonstration of a stimulatory effect of iron on the growth of insect cells.

Although the saturation densities reached by cells of both lines used in this study were exceptional, the population doubling times for the CL-15 cells were unusually high. Normal population doubling times for cells of this *S. frugiperda* line are 15–25 h (Weiss and Vaughn, 1986). The unusually long lag phase in the growth cycles observed in these studies can be overcome in most systems by increasing the inoculation level. Other physical factors such as rotation speed may also affect this growth parameter. These factors will need to be investigated.

The estimated cost of materials for this medium is less than $2.00 per liter. Other costs associated with the production of the medium could increase the total to $5 or $6 per liter which meets the criteria that were established when the project was begun. In an earlier study, Weiss and his colleagues (1992) reported the testing of a similar medium in which good yields of infectious virus were obtained. They estimated that this medium could be sold in high volume sales for $3 to $5 per liter.

Although the specific activity of the polyhedra produced in IBL10 medium was not equal to that of polyhedra produced in insects, the results are encouraging. IBL10 medium supported higher saturation densities with the two cell lines tested than any we have achieved previously. The projected costs of the medium are within the limits needed for *in vitro* production of virus and the polyhedra yields quantitatively equal to those with previous media were obtained.

References

Gardiner, G. R.; Stockdale, H. Two tissue culture media for production of lepidopteran cells and nuclear polyhedrosis viruses. J. Invertebr. Pathol. 25:363–370; 1975.

Goodwin, R. H.; Adams, J. R. Nutrient factors influencing viral replication in serum-free insect cell culture. In: Kurstak, E.; Maramorosch, K.; Dubendorfer, A., ed. Invertebrate systems *in vitro*. Amsterdam: Elsevier/North Holland Biomedical Press; 1980:493–509.

Lynn, D. E.; Dougherty, E. M.; McClintock, J. T., et al. Development of cell lines from various tissues of Lepidoptera. In: Kuroda, Y.; Kurstak, E.; Maramorosch, K., ed. Invertebrate and fish tissue culture. Tokyo: Japan Scientific Societies Press; 1988:239–242.

Lynn, D. E.; Hung, A. C. F. Development of continuous cell lines from the egg parasitoids *Trichogramma confusum* and *T. exiguum*. Arch. Insect Biochem. Physiol. 18:99–104; 1991.

Mitsuhashi, J. Nutritional requirements of insect cells *in vitro*. In: Mitsuhashi, J., ed. Invertebrate cell system applications. Vol. I. Boca Raton, FL: CRC Press, Inc.; 1989:3–20. Vail, P. V.; Jay, D. L.; Romine, C. L. Replication of the *Autographa californica* nuclear polyhedrosis virus in insect cell lines grown in modified media. J. Invertebr. Pathol. 28:263–267; 1976.

Vail, P. V.; Jay, D. L.; Romine, C. L. Replication of the *Autogrpha californica* nuclear polyhedrosis virus in insect cell lines grown in modified media. J. Invertebr. Pathol 28:263–267;1976.

Vaughn, J. L.; Fan, F. Low cost, serum-free medium for the production of baculoviruses *in vitro*. Fraser, M. J., Jr., ed. Proceedings of the Eighth International Conference on Invertebrate and Fish Tissue Culture; 1991 June 16–20; Anaheim, CA. Columbia, MD. Tissue Culture Association; 1991:111–116.

Vaughn, J. L.; Fan, F.; Daugherty, E. M., et al. The use of commercial serum replacements in media for the in vitro replication of nuclear polyhedrosis virus. J. Invertebr. Pathol. 58:297–304; 1991.

Vaughn, J. L.; Goodwin, R. H.; Tompkins, G. J., et al. The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera: Noctuidae). In Vitro 13:213–217; 1977.

Weiss, S. A.; Godwin, G. P.; Whitford, W. G., et al. Viral pesticides: *in vitro* process development. Proceedings of the 10th Australian Biotechnology Conference, 1992, February 4–7, Melbourne, Australia; Melbourne, Australia, Austral. Biotech. Assoc. 1992:67–71.

Weiss, S. A.; Smith, G. C.; Kalter, S. S., et al. Improved method for the production of insect cell cultures in large volume. In Vitro 17:495–502; 1981.

Weiss, S. A.; Vaughn, J. L. Cell culture methods for large-scale propagation of baculoviruses. In: Granados, R. R.; Federici, B. A., ed. The biology of baculoviruses, Vol. II. Boca Raton, FL: CRC Press, Inc.; 1986:63–87.